(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(71) Applicant *(for all designated States except US)*: **PHARMACIA & UPJOHN COMPANY** [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GURNEY, Mark, E.** [US/IS]; Tjnarnagata 39, Reykjavik (IS). **ABRAHAM, Irene** [US/US]; 2576 Ninth Avenue West, Seattle, WA 98119 (US).

(74) Agents: DELUCA, Mark et al.; Woodcock Washburn Kurtz Mackiewicz & Norris LLP, 46th Floor, One Liberty Place, Philadelphia, PA 19103 (US).

(54) Title: TRANSGENIC MOUSE MODEL OF HUMAN NEURODEGENERATIVE DISEASE
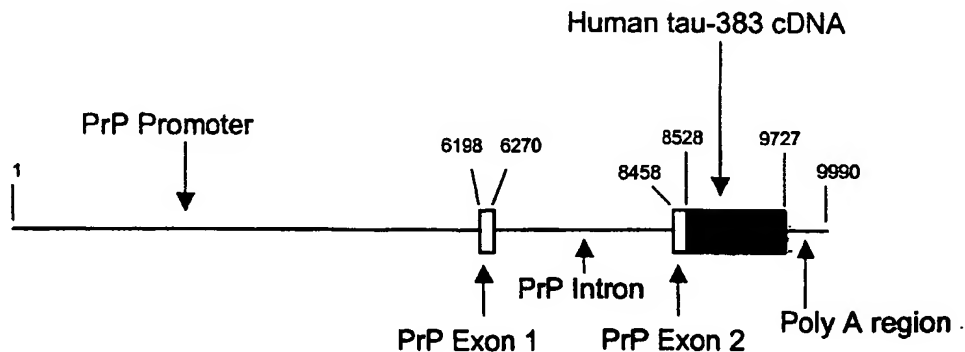
(57) Abstract: This invention provides transgenic mice expressing wild-type or mutant isoforms of human tau protein. The transgenic mice of the invention have many uses, including screening candidate drug compounds effective against neurodegenerative diseases and other diseases involving taupathies. The transgenic mice of the invention can also be used in genetic crosses with other transgenic mice strains to model the progression of neurodegenerative disease.

TRANSGENIC MOUSE MODEL OF HUMAN NEURODEGENERATIVE DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

5   This application claims priority to U.S. Provisional Patent Application, Serial Number 60/177,319 filed January 21, 2000, which is hereby incorporated by reference.

FIELD OF THE INVENTION

      This invention is related to the fields of transgenic animals, neurodegenerative

10   disease, and of identifying compounds to treat such diseases.

BACKGROUND OF THE INVENTION

      Many neurological diseases such as Alzheimer's disease, frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy,

15   corticobasal degeneration, and other undefined dementias, have the common pathological feature of neurofibrillary tangles (NFTs). NFTs are structures made up of paired helical filaments (PHFs), composed primarily of tau protein in a hyperphosphorylated state. Tau belongs to a family of microtubule associated proteins (MAPs) that are thought to play critical roles in stabilizing and remodeling the neuronal cytoskeleton. Under normal

20   physiological conditions tau is found primarily in the brain, associated with the cellular cytoskeleton.

      The adult human brain expresses six different isoforms of tau protein, ranging in size from 352 to 441 amino acid residues. The isoforms differ by having either three or four microtubule binding domains, and by the presence or absence of amino-terminal

25   inserts. Tau is phosphorylated in the brain by a variety of kinases, although the relationship between hyperphosphorylation and tangle formation is unclear. It is also

unclear how plaque and tangle formation leads to cognitive dysfunction and neuronal loss in patients with Alzheimer's disease.

Insight into the relationship of tau pathology and dementia has come through the study of FTDP-17, which is caused by mutations in the *tau* gene (see generally Goedert *et al.*, 1998, Neuron 21:955-958). The signature neuropathy associated with FTDP-17 is insoluble filamentous aggregates of hyperphosphorylated tau protein occurring in the absence of amyloid plaque and Lewy bodies, hallmarks of Alzheimer's disease and Pick's disease, respectively. Tau deposits are present not only in neurons, but also in large numbers of glial cells including predominantly oligodendrocytes, and also astrocytes (Spillantini *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:4113-4118). Many of the tau protein deposits also react with antibodies directed against ubiquitin. Immuno-electron microscopy demonstrates that tau is present as cytoplasmic filaments reminiscent of the paired helical filaments found in Alzheimer's disease, but these helical filaments have a different diameter and periodicity (Spillantini *et al.*, 1998, Am. J. Pathol. 153:1359-1363). Pathology varies between patients and may correlate with the particular *tau* mutation (Rizzu *et al.*, 1999, Am. J. Hum. Genet. 64:414-421).

Human tau protein has been expressed in transgenic mice in a number of studies. Gotz *et al.* (1995, EMBO J., 14:1304-1313) used the human Thy-1 promoter to express, in transgenic mice, the longest isoform of wild-type human tau, which is 441 amino acids in length, containing the 58 amino acid amino-terminal insert and four microtubule binding domains. Brion *et al.* (1999, Am. J. Pathol. 154:255-270) used the murine 3-hydroxy-methyl-glutaryl CoA reductase (HMG-CR) promoter to express the shortest human tau isoform, which is 352 amino acids in length, contains no amino-terminal inserts, and has three microtubule binding domains. An increase in tau phosphorylation at sites associated with Alzheimer's disease pathogenesis is seen in both models, as shown by reactivity with the PHF1 and AT8 monoclonal antibodies that recognize phosphoepitopes on the tau protein. In the HMG-CR/*tau* mice, this increase in tau phosphorylation is accompanied by the formation of a conformation-dependent epitope associated with early Alzheimer's disease pathology (Brion *et al.*, *supra*). This epitope is detected with an antibody designated Alz50, raised against paired helical filaments purified from Alzheimer's disease brain. Neurofibrillary tangles were not present in either transgenic model.

Zahr *et al.* (1999, Soc. Neurosci. 25:(A)447.1) have recently reported transgenic

expression in mice of mutant tau, containing the V337M mutation (*i.e.*, the valine at position 337 of the wild-type is replaced with methionine) in the context of a three microtubule binding domain isoform, under the regulatory control of the mouse prion promotor.

5   Additional transgenic models in which an increase in tau phosphorylation is seen include mice expressing mutant human amyloid precursor protein (APP) (Sturchler-Pierrat *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:13287-13292), glycogen synthase kinase-3beta (Brownlees *et al.*, 1997, Neuroreport 8:3251-3255), and the serine/threonine kinase Mos (James *et al.*, 1996, Neurobiol. Aging 17:235-241). Filamentous tau pathology is also

10   absent from these models.

Considerable effort has been put into making transgenic models of human neurodegenerative disease based on expression in transgenic mice of pathogenic human mutations. Choice of mutation, choice of transgene vector, expression in appropriate types of neurons, and transgenic expression of the mutant human protein at sufficient levels are

15   all critical to success. Strategies for creation of transgenic models have therefore varied widely. For example, the transgenic models for familial amyotrophic lateral sclerosis used the 12 Kb SOD1 gene for expression of human Cu,Zn superoxide dismutase containing the mutations G93A (Gurney *et al.*, 1994, Science 264:1772-1775) or G37R (Wong *et al.*, 1995, Neuron 14:1105-1116).

20   Transgenic models for amyloid β-peptide deposition in Alzheimer's disease have used either the platelet-derived growth factor promoter (Games *et al.*, 1995, Nature 373:523-527), insertion into the human prion gene (Hsiao, 1997, J. Neural. Transm. Suppl. 49:135-144), or the human Thy-1 promoter (Sturchler-Pierrat *et al.*, *supra*) to elicit transgenic expression of the amyloid precursor protein, containing either the "London" or

25   "Swedish" mutations associated with familial, early-onset Alzheimer's disease. These types of transgenic mice are becoming extraordinarily valuable to the pharmaceutical industry for their use as "gatekeeper" models for preclinical evaluation of promising drug candidates. An example of such a use was the testing of riluzole in the transgenic model of amyotrophic lateral sclerosis, where it proved to have a beneficial effect (Gurney *et al.*,

30   1996, Ann. Neurol. 39:147-157). The drug later became the first to be approved by the Food and Drug Administration for the treatment of the human disease. Similar use is

being made of transgenic mice that model various aspects of Alzheimer's disease pathology.

The current transgenic models of Alzheimer's disease, which are based on expression of mutant human amyloid precursor protein, are incomplete models of the disease. Although they show amyloid β-peptide deposition in plaque, no neurofibrillary tangles are noted, nor is there extensive neuronal cell loss or consequent brain atrophy. Thus, the art is in need of new and/or better models of Alzheimer's disease as well as other neurodegenerative diseases. The present invention is directed to addressing these, and other needs.

SUMMARY OF THE INVENTION

This invention provides transgenic mice expressing wild-type or mutant isoforms of human tau protein. The transgenic mice of the invention can be used for screening candidate drug compounds, which may be effective against neurodegenerative diseases and other diseases involving taupathologies. The transgenic mice of the invention can also be used in genetic crosses with other transgenic mice strains to model the progression of neurodegenerative disease.

In one aspect, the present invention is directed to a transgenic mouse expressing a human tau protein under the regulatory control of the mouse prion gene promoter. The human tau protein that is expressed in the transgenic mouse may be either wild-type or may contain a mutation. The mutation may be one previously identified in humans, or may be a novel mutation. The human tau protein expressed may be any of the six different isoforms.

The invention is also directed to the use of the transgenic mice of the invention as models of neurodegenerative disease.

In another aspect of the invention, the transgenic mice are used to discover drugs that modulate tau protein expression and/or activity.

These and other aspects of the invention are more fully described below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic of a *tau* transgene construct used to prepare a

transgenic mouse of the present invention. See also Table 3, *infra*.

Figure 2 shows immunoblot analyses of human tau protein expression in the brains of wild-type and two kinds of mutant *tau* transgenic mice. Mouse brain samples are

5   identified by mouse transponder ID number (5 digit number) or by ear tag number (337 and 341) (see Table 4, *infra*). Samples 21080, 21089, 21096, 22410, 22418, and 22435 serve as controls without human tau protein expression, and are from non-transgenic litter mates of the transgenic mice. Mice with transponder numbers are the progeny of the original founder mice mated with (C57Bl/6 x SJL)F1 partners; mice with three digit

10   numbers are the founder mice.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides transgenic mice, containing transgenes of human *tau*, and expressing human tau protein.

15   Transgenic animals contain within their genetic material some genetic information from another organism. For example, a transgenic mouse might have a human gene inserted into its genetic material. Such foreign genetic material is referred to as a transgene.

Transgenic mice can be generated in a number of manners known to those of skill

20   in the art. Typically, a transgenic animal is generated according to the following basic steps: 1) engineer a nucleic acid construct with a promoter (regulatory sequences) and cDNA or gene sequences of interest; 2) linearize and inject the transgene construct into the pronuclei of embryos; 3) implant the embryos into the uterus of a pseudo-pregnant female; 4) screen the offspring that are born (the live pups in the case of mice) for the presence of

25   the transgene; and 5) breed the transgene-positive pups (founders) to get transgenic "lines." Examples of these and related techniques are found in, *e.g.*, Hogan, B., Costantini, F. & Lacy, E., eds., *Manipulating the Mouse Embryo : A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1986), which is incorporated herein by reference in its entirety.

30   Tau is predominantly expressed in neurons; the protein is principally localized in the axons. Some expression has also been noted in oligodendrocytes and astrocytes. Six different isoforms of tau protein are expressed in the adult human brain. Each isoform is

generated by differential splicing of an RNA transcript from the single *tau* gene, located

on chromosome 17. The six isoforms range in size from 352 to 441 amino acid residues.

The isoforms differ by having either three or four microtubule binding domains (imperfect

repeats of 31 or 32 amino acid residues) in the carboxy-terminus, and by the presence or

5   absence of 29- or 58-amino acid inserts, of unknown function, in the amino-terminus.

Tau protein microtubule binding domains, which contain the core microtubule

binding domain motif proline-glycine-glycine-glycine (PGGG), are designated R1, R2,

R3, and R4, and are encoded by exons 9, 10, 11, and 12, respectively. Exon 10, which

encodes amino acid residues 275 through 305, is alternatively utilized (present in three of

10   the six isoforms), such that the R2 microtubule binding domain is present only in tau

isoforms containing four repeats. Thus, the isoforms range in size from 352 amino acid

residues (with no amino-terminal inserts and three microtubule binding domains) to 441

amino acid residues (with a 58 amino acid amino-terminal insert and four microtubule

binding domains). Table 1 summarizes the features of the six human tau isoforms, which

15   are designated "A" through "F." The isoforms may also be respectively referred to as

numbers "1" through "6," or by their total length in amino acid residues.

Table 1: The Six Isoforms of Human Tau Protein:

		N-Terminal Insert	Microtubule Binding Domains	Length (amino acids)
20				
	A	none	3	352
	B	29 amino acids	3	381
	C	58 amino acids	3	410
	D	none	4	383
25	E	29 amino acids	4	412
	F	58 amino acids	4	441

In fetal brain, only the smallest isoform of tau ("A" in Table 1) is expressed. The

amino acid residues in tau protein, regardless of isoform, are typically referred to by the

30   position number they would have in the largest isoform ("F"), which has 441 residues.

See Spillantini & Goedert, 1998, Trends Neurosci. 21:428-433.

The DFTP-17 associated mutations in *tau* provide an important entry point in the

study of neurofibrillary tangle formation as it relates to neuronal dysfunction and death. *Tau* mutations linked to FTDP-17 are either missense mutations (see Table 2), leading to substitution of one amino acid for another, or mutations in the 5' splice site of exon 10.

Most of the missense mutations associated with FTDP-17 occur in or near three of

5   the four homologous microtubule binding domains within tau. Many of the missense mutations reduce the ability of tau to bind microtubules and to promote microtubule assembly in biochemical assays (Hong *et al.*, 1998, Science 282:1914-1917; Hasegawa *et al.*, 1998, FEBS Lett. 437:207-210). The arginine to tryptophan mutation at amino acid 406 (R406W), although linearly distant from a microtubule binding domain (residue 406

10   is encoded in exon 13, which does not contain a repeat), is believed to interfere with microtubule binding through a conformational change in the protein (Hong *et al.*, *supra*). The asparagine to lysine at 279 (N279K), proline to leucine at 301 (P301L), and serine to asparagine at 305 (S305N) mutations are seen only in four repeat-containing tau isoform proteins, as these residues are encoded by alternatively spliced exon 10. The valine to

15   methionine at residue 337 (V337M) mutation is of particular interest, because it causes a tau pathology in the brain indistinguishable from that seen in Alzheimer's disease (Spillantini *et al.*, 1996, Acta Neuropathol. 92:42-48; Poorkaj *et al.*, 1998 Ann. Neurol. 43:815-825).

20   **Table 2: *Tau* Missense Mutations Associated with FTDP-17**

Codon*	Amino Acid Substitution	Location Within Tau Protein	Reference
272	Glycine → Valine (G272V)	R1 Microtubule Binding Domain	1
279	Asparagine → Lysine (N279K)	R1 Microtubule Binding Domain	2, 3, 7
301	Proline → Leucine (P301L)	R2 Microtubule Binding Domain	1, 2, 4, 7
305	Serine → Asparagine (S305N)	R2 Microtubule Binding Domain	3, 8
337	Valine → Methionine (V337M)	R3 Microtubule Binding Domain	5, 6, 7

406	Arginine → Tryptophan (N406W)	Carboxy-Terminal to R4 Microtubule Binding Domain	1, 7

* Numbering is based on the longest tau isoform, containing 441 amino acid residues.

1. Hutton *et al.*, 1998, Nature 393:702-705

2. Clark *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95:13103-13107

5 3. Hasegawa *et al.*, 1999, FEBS Lett. 443:93-96

4. Dumanchin *et al.*, 1998, Hum. Mol. Genet. 7:1825-1829

5. Poorkaj *et al.*, *supra* (refers to codon based on numbering of a shorter tau isoform)

6. Spillantini *et al.*, 1998, Brain Pathol. 8:387-402

7. Hong *et al.*, *supra*

10 8. Iijima *et al.*, 1999, Neuroreport 10:497-501

Two studies have examined the effect of several tau mutants on microtubule structure after transfection of the *tau* genes into cells in culture (Dayanandan *et al.*, 1999, FEBS Lett. 446: 228-232; Arawaka *et al.*, 1999, Neuroreport 10:993-997). These studies

15 show variable effects of the mutants on microtubule structure. Arawaka *et al.* (*supra*) showed that the presence of the V337M mutation results in fewer cell processes and reduced microtubule networks. Dayanandan *et al.* (*supra*) reported no differences in morphology with V337M, P301L or R406W, but did see differences in cell processes after treatment with cytochalasin B. In addition, Nacharaju *et al.* (1999, FEBS Lett. 447:195-

20 199) have shown that mutants P301L, V337M and R406W cause an accelerated aggregation of tau into filaments *in vitro*.

A number of splice mutations have been identified in association with FTDP-17. These mutations are located in the 5' region of the intron immediately following exon 10 of the *tau* gene. The mutations are: G to A at nucleotide position +3 of the intron

25 (Spillantini *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95:7737-7741); A to G at +13 (Hutton *et al.*, 1998, Nature 393:702-705); C to T at +14 (Hutton *et al.*, *supra*, Clark *et al.*, *supra*, Hong *et al.*, *supra*); and C to T at +16 (Hong *et al.*, *supra*). These splice mutations all destabilize a potential stem-loop structure that may be involved in regulation of the alternative splicing of exon 10. This causes more frequent usage of the 5' splice site and

an increased proportion of *tau* transcripts that include exon 10 (Hutton *et al.*, *supra*). The increase in transcripts containing exon 10 presumably is mirrored by an increase in the proportion of tau protein containing four repeats of the microtubule binding domain. An increase in splicing of exon 10 is also seen with two of the tau missense mutations,

5   asparagine to lysine at residue 279 (N279K) and serine to asparagine at residue 305 (S305N), neither of which alters microtubule binding or assembly (Hasegawa *et al.*, 1999, *supra*).

Based on what is understood about the mutations associated with FTDP-17, two types of mutations are sufficient to cause tau neuropathology: (1) mutations that alter the

10   ability of tau to bind to microtubules and promote microtubule assembly; and (2) mutations that increase the proportion of tau protein containing four microtubule binding domains.

The present invention provides transgenic mice expressing human tau protein in either wild-type or mutant forms. In one embodiment of the invention, human tau protein

15   is expressed in transgenic mice under the control of regulatory sequences from the mouse prion (PrP) gene promoter region. Preferably such expression is directed to the brain. More preferably, the regulatory sequences include the 5' flanking sequence of the mouse prion gene promoter, the first exon, the first intron, and the initial noncoding portion of the second exon of the mouse prion gene.

20   In a preferred embodiment, the human tau protein that is expressed in the transgenic mice is the wild-type 383 amino acid isoform of the protein (isoform D). This isoform contains four microtubule binding domains (*i.e.*, it utilizes exon 10), but has no amino-terminal insert; it is the shortest of the four repeat-containing isoforms.

In another embodiment of the invention, mutant human tau protein is expressed in

25   the transgenic mice of the invention. Preferably, the mutation is the V337M mutation in the 383 amino acid isoform of the human tau protein. Those of skill in the art will recognize that other mutations can be used in the context of any of the six different isoforms of tau.

The tau protein expressed in the transgenic mice of the invention may contain a

30   mutation known to be associated with FTDP-17, including, but not limited to, the G272V, N279K, P301L, S305N, V337M, and R406W missense mutations.

The invention also provides transgenic mice expressing mutant forms of human tau

protein, containing mutations other than those known to be associated with FTDP-17.
Those of skill in the art will recognize that such mutations can be selected based on the
locations of microtubule binding domains and other important features of the tau protein.
Mutations to be incorporated into *tau* transgenes can be chosen based on predicted

5　interference with tau function or analogy with disease-associated mutations. Such
mutations can be tested for their effect on tau function by transfection studies on tissue
culture cells. Those of skill in the art will recognize that any mutations in *tau* which
modulate tau activity can be used in the transgenic mice of the invention.

　　　Examples of amino acid residues in tau that can be mutated include residue 332,

10　which is a proline in wild-type tau. Residue 332 is located in the core PGGG motif of the
R3 microtubule binding domain. Mutation of residue 332 from proline to leucine
(P332L), mimics the FTDP-17-associated alteration at residue 301 (P301L) in the core
PGGG motif of the R2 microtubule binding domain. In a preferred embodiment of the
invention, the mutant human tau expressed in the transgenic mice contains the P332L

15　mutation in the 383 amino acid isoform of the protein.

　　　A further embodiment of the invention includes transgenic mice expressing human
tau protein containing a missense mutation at amino acid residue 364, where proline has
been changed to leucine, resulting in alteration of the core PGGG motif of the R4
microtubule binding domain, also mimicking the FTDP-17-associated mutation, P301L.

20　　　In a preferred embodiment of the invention, the nucleotide sequence encoding the
human tau protein is provided by a nucleic acid containing no intronic sequences. Other
embodiments may include intronic sequences in the transgene to study the effect of
mutations that occur in such intronic regions, for example, the mutation of G to A at
position +3 in the intron immediately following exon 10. Thus, the invention also

25　provides transgenic mice containing transgenes of human *tau* with intronic mutations.

　　　In another embodiment, the transgenic mice of the invention are used as models of
the progression of neurodegenerative disease or other diseases whose hallmark is neuronal
cell death. Preferably, the transgenic mice of the invention will develop, with age, tau
pathologies including tau hyperphosphorylation and filamentous aggregates of tau. Tau

30　hyperphosphorylation may be revealed by immunostaining for tau phosphoepitopes such
as those recognized by PHF1 or AT8 monoclonal antibodies. Filamentous tau
neuropathology may be revealed by staining for ubiquitin immunoreactivity. More

preferably, the tau transgenic mice will display neuronal loss and cognitive dysfunction.

In another embodiment of the invention, transgenic mice expressing tau protein are used to screen for compounds that can be used to treat neurodegenerative disease.

It will be appreciated by those of skill in the art that the transgenic mice of the
5   present invention are useful for analysis of neurodegenerative diseases involving tau pathologies. Such diseases involving tau pathology, collectively referred to as taupathies, include, but are not limited to, Alzheimer's disease, Pick's disease, FTDP-17, progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD), Down's syndrome, Argyrophilic grain disease, amyotrophic lateral sclerosis/parkinsonism dementia complex
10   of Guam, Non-Guamanian motor neurone disease with NFT, Niemann-Pick disease type C, subacute sclerosing panencephalitis, postencephalitic parkinsonism, dementia pugilistica, myotonic dystrophy, Gerstmann-Straeussler-Scheinker disease with tangles, prion protein amyloid antipathy, presenile dementia with tangles and calcifications, and Hallervorden-Spatz disease (Tolnay & Probst, 1999, Neuropathol. Appl. Neurobiol. 3:171-
15   187).

Thus, in another embodiment, the invention is a model of neurodegenerative disease comprising any of the above described transgenic mice. In another embodiment, the transgenic mice of the invention are used in the screening of drug compounds to identify those capable of treating neurodegenerative disease.

20   In another embodiment, the transgenic mice of the invention are used in the screening of drug compounds to identify those capable of modulating the expression and/or activity of tau protein.

In another embodiment, the transgenic mice of the invention are used to screen for compounds that can be used to treat diseases that involve neuronal cell death.

25   Other embodiments of the invention will be readily understood by those of skill in the art.

The invention is further illustrated by way of the following examples, which are intended to elaborate several embodiments of the invention. These examples are not intended, nor are they to be construed, as limiting the scope of the invention. It will be
30   clear that the invention may be practiced otherwise than as particularly described herein. Numerous modifications and variations of the present invention are possible in view of the teachings herein and, therefore, are within the scope of the invention.

EXAMPLES

Example 1: Preparation of Transgenic Vectors

Plasmid phgPrP, containing most of the mouse *PrP* gene 5' end and its 3' flanking

5   sequences, was obtained from Marek Fischer, Department of Medicine, Division of

Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, Ramistrasse

100, CH-809 Zurich, Switzerland. Plasmid T43, containing the cDNA encoding the wild-

type 383 amino acid isoform of human tau [SEQ ID NO:1] in the vector pSG5

(Stratagene, La Jolla CA; GenBank Accession AF013258), was obtained from Virginia

10   Lee, Department of Pathology and Laboratory Medicine, University of Pennsylvania

School of Medicine, 34th & Spruce Street, Philadelphia, PA 19104.

The sequence of the phgPrP insert was confirmed by the DNA Sequencing Core

(Pharmacia & Upjohn). The 14,214 base pair (bp) phgPrP insert sequence was verified by

comparison with the 38,418 bp GenBank sequence deposit for the *Mus musculus* short

15   incubation prion protein *Prnpa* gene (Accession Number U29186). The phgPrP insert

sequence extends the GenBank deposit by 5,047 bp of 5' flanking sequence [SEQ ID

NO:2]. The entire phgPrP insert was cloned into pBluescript KS+ (Stratagene, La Jolla,

CA). The resulting construct was opened with KpnI to remove the *PrP* coding sequence

and 3' flanking region of the mouse *PrP* gene. Amplification by polymerase chain

20   reaction (PCR) was used to resynthesize the 3' mouse *PrP* flanking sequence such that it

contained 5' KpnI and XhoI sites for cloning. This created the vector 97-1, used for

subsequent steps in assembly of the *PrP/tau* transgene.

To assemble the *PrP/tau* transgene, the 97-1 vector was opened with XhoI and

SalI. This retained the mouse *PrP* promoter and sequences through the initial, noncoding

25   portion of *PrP* exon 2. SalI was used to excise the human *tau*-383 insert fused to the

polyadenylation site from the pSG5 vector. After dephosphorylation of the 97-1 vector,

the two DNAs were ligated and transformed into *E. coli* strain DH5α (GIBCO/BRL,

Gaithersberg, MD). Bacterial colonies were screened for directional ligation of the *tau*

insert in the sense orientation by PCR using the primers

30   AGTAATTGAAAGAGCTCAGACGATG [SEQ ID NO:3] and

TGTCACCCTCTTGGTCTTGGTGC [SEQ ID NO:4].

The V337M mutation was introduced into the *tau*-383 coding sequence by

12

mutagenesis of a 302 bp PstI-HindIII DNA fragment using PCR. The PCR reaction was

carried out using: a primer positioned 5' of the PstI site having the sequence

GTACTCCACCCAAGTCGCCGTC [SEQ ID NO:5], a short 3' reverse primer containing

the HindIII site having the sequence GCAGCAGCATCGAAGCTTCTCAG [SEQ ID

5   NO:6], and a longer reverse "patch" primer containing the V337M mutation having the

sequence GCAGCAGCATCGAAGCTTCTCAGATTTTACTTCCATCTGGCCACCTCC

[SEQ ID NO:7].

The P332L mutation was also introduced into the *tau*-383 coding sequence by

mutagenesis using PCR. The PCR reaction was carried out using a forward primer having

10   the sequence CCGCCAAGAGCCGCCTGCAG [SEQ ID NO:8], the SEQ ID NO:6

reverse primer, and a longer reverse "patch" primer containing the P332L mutation,

having the sequence

GCAGCAGCATCGAAGCTTCTCAGATTTTACTTCCACCTGGCCACCTCCTAGTTT

ATGATGG [SEQ ID NO:9].

15   The PCR reaction was performed with an Advantage-HF™ high-fidelity PCR kit

(Clontech, Palo Alto, CA), using the buffer supplied with the kit. The "patch" PCR

primer was used at 1/100th the concentration of the short, flanking primers. Five cycles of

(95°C for 45 seconds, 60°C for 2 minutes, 72°C for 3 minutes) followed by 20 cycles of

(95°C for 45 seconds, 68°C for 3 minutes) were used for synthesis of the mutant fragment.

20   To construct a mutant *tau*-383 insert, the PstI-HindIII fragment from wild-type *tau*-383

was exchanged for a mutant fragment. Proper introduction of mutations was confirmed by

sequencing in the Pharmacia & Upjohn DNA sequencing core. The cDNA sequence for

the V337M mutant of the 383 amino acid isoform is provided in SEQ ID NO:10. The

cDNA sequence for the P332L mutant of the 383 amino acid isoform is provided in SEQ

25   ID NO:11. The same procedure as described above for wild-type *tau* was used to

assemble mutant inserts, *tau*-V337M and *tau*-P332L, into the *PrP* transgene vector. Table

3 presents the general features in the transgene constructs used to generate *tau* transgenic

mice. The features correspond to those depicted in Figure 1. The sequence for the entire

9990 nucleotide sequence of the *PrP/tau* transgene vector comprising wild-type 383 *tau* is

30   provided in SEQ ID NO:15.

13

Table 3. General Features in the *PrP/tau* Transgene Vectors Used for Microinjection

Feature	Nucleotide Location	
PrP promoter	1-6197	(Nucleotides 1-5047 are designated SEQ ID NO:2)
PrP exon 1	6198-6270	
PrP intron 1	6271-8457	
PrP exon 2	8458-8527	
tau-383 cDNA	8528-9727	
poly(A) region	9728-9990	

Example 2: Generation and Screening of Transgenic Mice

The 10 Kb plasmid inserts were prepared for microinjection by the University of Michigan Transgenic Facility following standard protocols. They were injected into F2 fertilized mouse eggs from matings of F1 hybrid C57Bl/6 x SJL mice. Progeny were genotyped by Taqman™ PCR using mouse genomic DNA prepared from tail biopsies using the Qiagen tissue kit according to manufacturer's instructions. Amplification was performed with the Taqman™ PCR Core Reagents Kit (PE Biosystems) using 50mM KCl, 10mM Tris-HCl, pH 8.3, 10mM EDTA, 60nM Passive Reference Dye 1, 5mM $MgCl_2$, 200µM each of dATP, dGTP and dCTP, 400µM dUTP, 200nM each primer, 100nM Taqman probe, 0.5 units uracil-N-glycosylase, and 1.25 units AmpliTaq Gold. Specific amplification of human *tau* DNA was performed using the primers G29-Fp GCATTGGAGACACCCCCAG [SEQ ID NO:12], G29-Rp GCTTTTACTGACCATGCGAGC [SEQ ID NO:13], and G29 Taqman™ probe with 5' and 3' colorimetric tags FAM-CTGGAAGACGAAGCTGCTGGTCACG-TAMRA [the nucleotide portion of which is designated SEQ ID NO:14] with the thermal cycling parameters, 50°C-2 minutes, 95°C-10 minutes, 40 cycles of [95°C-15 seconds, 60°C-1 minute], and 25°C hold. The starting amount of mouse genomic DNA template was 10ng. A standard curve was generated ranging 5 orders of magnitude from 10^2 to 10^6 copies of linearized *PrP/tau* transgene plasmid DNA, which was diluted into mouse genomic DNA to give a final concentration of 10ng/µl. All reactions were performed in duplicate.

Multiple mice were generated from fertilized mouse eggs injected with wild-type

or mutant constructs. For each construct, several founder mice, carrying the *tau* transgene, were identified. Table 4 shows that the transgenic mice contained varying numbers of copies of the *PrP/tau* transgene, from which varying levels of transcript were expressed.

5   **Table 4: Summary of Analysis of Transgenic *Tau* Mice**

Transgene and founder mouse number	Mouse Id	Gene Copy #	*htau* RT-PCR levels copies/67ng total RNA positive RT sample	*htau* RT-PCR levels copies/67ng total RNA negative RT sample
TgN(*PrP/tau*) 355	21088	4.6	16,599	81
TgN(*PrP/tau*) 327	21097	2.6	5,402	13
TgN(*PrP/tau*) 326	21083	13.7	595	161
	21214	10.6	754	26
TgN(*PrP/tau-*V337M) 249	22419	2.4	33,671	6
	22420	2.6	26,912	20
TgN(*PrP/tau-*V337M) 250	22405	0.3	19,138	0
	22408	0.2	21,814	0
TgN(*PrP/tau-*V337M) 257	22436	0.9	10,755	2
	22437	0.2	8,842	1
TgN(*PrP/tau-*P332L) 337	337	16.8	ND[1]	ND
TgN(*PrP/tau-*P332L) 341	341	1.4	ND	ND

[1]Not determined.

Table 4 summarizes the analysis of the transgenic mice. The first column lists the transgene designation (TgN) and founder mouse number. The founder mice are indicated

10   by the numbers 355, 327, 326, 249, 250, 257, 337 or 341, after the transgene name TgN(*PrP/tau*), TgN(*PrP/tau*-V337M) or TgN(*PrP/tau*-P332L). The second column lists the mouse identification (Id) number (either a 5-digit transponder number or a 3-digit founder number, as used in Figure 2). Mice with a 5-digit number are the progeny of the founder mouse indicated in the first column by the three digit number after the transgene

15

name. The third column lists the haploid copy number of the human *tau* (*htau*) transgene, based on DNA levels derived after PCR analysis. This number was measured either in the founder or founder progeny, as indicated in the second column. The fourth column lists the human *tau* (*htau*) transgene mRNA expression, as determined by reverse transcriptase-

5   PCR (RT-PCR). The fifth column lists the background amplification of the RNA samples, without conversion to cDNA by RT.

Example 3: Human Tau Expression

Mouse brain samples were harvested after intracardiac perfusion with cold

10   phosphate-buffered saline, for analysis of human *tau* mRNA and tau protein expression. For cardiac perfusion, mice were deeply anesthetized, then perfused transcardially until the effluent was cleared of blood. Tissue samples were frozen immediately on dry ice, then stored at -70°C until analysis.

Total RNA was prepared from homogenized cortical brain tissue using the RNeasy

15   mini kit (Qiagen). The RNeasy protocol was modified by the addition of the RNase-free DNase set protocol (Qiagen) to treat the RNA, while bound onto the column, with RNase-free DNase I at room temperature for 15 minutes to 1 hour, to remove any residual genomic DNA contamination. Two micrograms of total RNA was converted to cDNA in a 30µl reaction containing, 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$, 500µM

20   each dNTP, 0.5µg random hexamers, 30 units RNase inhibitor, and 400 units Superscript II reverse transcriptase (RT), which was incubated at 42 °C for 1 hour. The Superscript II RT enzyme was then heat inactivated by incubation at 95°C for 5 minutes. To assess the extent of genomic DNA contamination in the total RNA samples, control reactions, without RT, were also performed for each sample.

25   For determination of human *tau* mRNA expression, PCR amplification on the ABI PRISM 7700 instrument was performed using 2X Taqman™ universal PCR master mix (PE Biosystems) containing 200nM G29Fp and 200nM G29Rp primers and 100nM G29 Taqman probe. The final PCR reaction volume was 25µl, and contained 5µl of a 1:5 dilution of the cDNA reaction described above. The thermal cycling parameters were as

30   follows, 50°C-2 minutes, 95°C-10 minutes, 40 cycles of [95°C-15 seconds, 60°C-1 minute], and 25°C hold. A relative standard curve was generated ranging 5 orders of magnitude

from 10^2 to 10^6 copies of linearized *PrP/tau* transgene plasmid DNA, which was diluted into mouse genomic DNA to a final concentration of $10ng/\mu l$. All samples were run in either duplicate or triplicate.

For determination of human tau protein expression by immunoblot analysis, brain

5   tissue was minced in $600\mu l$ lysate buffer (0.1M MES, pH 6.8, 0.75M NaCl, 0.5mM EGTA, 2mM DTT, 2mM PMSF, $10\mu g/ml$ aprotinin, $10\mu g/ml$ leupeptin, $10\mu g/ml$ soy bean trypsin inhibitor, $5\mu g/ml$ pepstatin) on ice. Brain fragments were ground with 10 strokes of a pestle in a pre-chilled TenBroeck tissue homogenizer. Lysate was centrifuged at 40,000g for 15 minutes at 4°C. Protein concentration of supernatants was determined by

10 Bio Rad protein assay, and lysates were stored at -80°C until used.

$50\mu g$ samples of the brain lysates were diluted into 4X gel loading buffer (250mM Tris-HCl, pH 6.8, 8% SDS, 20mM EDTA, 40% glycerol, 0.02% bromophenol blue, 20% β-mercaptoethanol), heated at 100°C for 5 minutes, and electrophoretically separated on a 7.5% polyacrylamide gel in running buffer (25mM Tris base, 192mM glycine, 0.01%

15 SDS). The proteins were transferred to Nitrobind nitrocellulose membrane (Micron Separations Inc, Westborough, MA) in transfer buffer (25mM Tris base, 192mM glycine, 20% methanol). The membranes were blocked for 1 hour in PBS containing 5% nonfat dry milk. Blots were probed overnight at 4°C with either the T14 or HT7 anti-human tau antibody (Zymed Laboratories, Inc., So. San Francisco, CA), diluted to $2\mu g/ml$ in a

20 solution of 10mM Tris-HCl, pH 7.5, 500mM NaCl, and 0.1% Tween 20 (TNTween), plus 1% BSA and 3% dry milk. After washing in TNTween, the blots were incubated for 1 hour at room temperature with goat anti-mouse, horseradish peroxidase-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) at 1:5000 dilution. The immunoblots were washed again in TNTween, treated with ECL

25 reagents for 1 minute, and exposed to X-ray film.

Progeny of the transgenic founders (mated to F1 hybrid (C57Bl/6 x SJL) partners) or the founders were analyzed for *PrP/tau* transgene expression in the brain. Both human *tau* mRNA (Table 4) and protein (Figure 2) were detected. Efficient, relatively high levels of human tau protein expression were obtained in transgenic mice using a mouse *PrP* gene

30 vector coupled to a heterologous human *tau*-383 cDNA. Roughly equivalent levels of protein expression were obtained from wild-type and mutant *PrP/tau* transgenes,

suggesting that expression of mutant tau protein is relatively well tolerated in the mouse. The levels of expression achieved appear to be higher than those reported by Gotz *et al.*, *supra* or Brion *et al.*, *supra*, although different antibodies and methods of analysis were used to establish levels of protein expression. Ranking by protein expression roughly

5   correlates with the PRISM measurement of mRNA expression.

Example 4: Microtubule Organization in GFP-*tau* Transfected Cells

To investigate the effect of mutations in the microtubule binding domains on tau protein interaction with microtubules, human embryonic kidney HEK293 cells were

10   transiently transfected with cDNA encoding the 383 human tau isoform fused to green fluorescent protein (GFP). Wild-type and the P332L, V337M, and P301L *tau* mutants were analyzed. Fluorescence and biochemical analyses showed that GFP-labeled mutant tau proteins P332L, V337M, and P301L have an altered intracellular distribution, as compared to GFP-wild type tau protein.

15   Studies using an anti-tubulin antibody revealed that GFP-wild-type tau protein co-localizes with microtubules. Some tau protein is also present in the cytoplasm, not associated with microtubules. In cells expressing high levels of wild-type tau, a reorganization of the microtubule cytoskeleton was observed. Bundles of microtubules could be seen, often in the form of a spiral or a ring around the circumference of the cell.

20   This is a phenomenon previously noted in non-neuronal cells expressing tau. Cells expressing low levels of GFP-wild type tau protein, contained a fine network of microtubules, often emerging from microtubule organizing center.

Cells expressing GFP-labeled mutant tau proteins P332L, V337M, and P301L exhibited higher levels of fluorescence dispersed in the cytoplasm than did cells

25   expressing GFP-wild type tau protein. The microtubule bundling and the microtubule network, seen in GFP-wild type tau expressing cells, was also reduced in cells expressing the mutant tau proteins.

Immunoblotting analyses of cytosolic and cytoskeletal cellular fractions confirmed the fluorescence studies, showing that cells expressing the GFP-mutant tau proteins

30   contained more tau protein in the cytosol than did cells expressing GFP-wild type tau. The difference was most dramatic for P332L, but the studies have confirmed that these

microtubule binding domain mutations interfere with tau protein interaction with microtubules.

Example 5: Cellular Analysis of Tau Transgenic Mice

5   General pharmacokinetic studies are used to analyze human tau expression in the transgenic mice. Pharmacokinetic studies include determination of the brain distribution of tau protein expression, and kinase activity assays. The state of phosphorylation of the tau protein is detected using monoclonal antibodies to specific phosphoepitopes of tau, including PHF1 and AT8, and various polyclonal antibodies. Filamentous tau

10   neuropathology is revealed by staining for ubiquitin immunoreactivity.

Example 6: Analysis of Neurodegenerative Disease in Transgenic Mice

Neurodegenerative disease is studied in *tau* transgenic mice by use of a variety of behavioral studies, including the Morris water maze test, mobility and gait tests, and

15   behavioral observation (see, *e.g.*, Moechars et al., 1999, Biol. Chem. 274:6483-6492).

Example 7: Drug Screening in Transgenic Mice

The transgenic mice are used for the screening of compounds that affect tau hyperphosphorylation, filament formation,or neurodegenerative disease criteria.

20   Compounds that inhibit kinase activity, protease activity, phosphatase activity, oxidative stress reactions, apoptosis, or protein aggregation are tested for their ability tomodulate 1) tau phosphorylation, 2) tau aggregate formation, 3) formation of cellular inclusions of paired helical filaments of tau, and 4) formation of neurofibrillary tangles in the transgenic mice. Those compounds exhibiting positive effects in such tests may be useful in the

25   treatment of neurodegenerative diseases.

All references cited herein are incorporated herein in their entirety by reference.

We claim:

1.   A transgenic mouse, comprising a transgene, said transgene comprising a polynucleotide encoding a human tau protein operably linked to at least a portion of a regulatory region of a mouse prion gene, wherein said transgenic mouse expresses human tau protein.

2.   The transgenic mouse of claim 1, wherein said human tau protein is selected from the group consisting of isoforms 352, 381, 410. 383, 412, and 441.

3.   The transgenic mouse of claim 2, wherein said human tau protein is isoform 383.

4.   The transgenic mouse of claim 1, wherein said human tau protein is selected from the group of mutants consisting of G272V, N279K, P301L, S305N, V337M, and R406W.

5.   The transgenic mouse of claim 4, wherein said human tau protein is a V337M mutant of isoform 383.

6.   The transgenic mouse of claim 2, wherein said human tau protein is further selected from the group of mutants consisting of G272V, V337M, and R406W.

7.   The transgenic mouse of claim 3, wherein said human tau protein is further selected from the group of mutants consisting of N279K, P301L, and S305N.

8.   The transgenic mouse of claim 2, wherein said human tau protein is mutant P332L.

9.   The transgenic mouse of claim 1, wherein said human tau protein is expressed in the brain.

20

10. The transgenic mouse of claim 1, wherein said regulatory region comprises said prion gene promoter and 5' flanking sequence, the first *PrP* exon, the first *PrP* intron, and the initial, noncoding portion of the second *PrP* exon.

11. A model of neurodegenerative disease comprising a transgenic mouse as in any of claims 1-10.

12. The model of neurodegenerative disease of claim 11 for use in the screening of drugs to treat said disease.

13. The model of neurodegenerative disease of claim 11 for use in genetic crosses to generate models of Alzheimer's disease.

14. A method of screening for a drug that modulates hyperphosphorylation of tau comprising the steps of
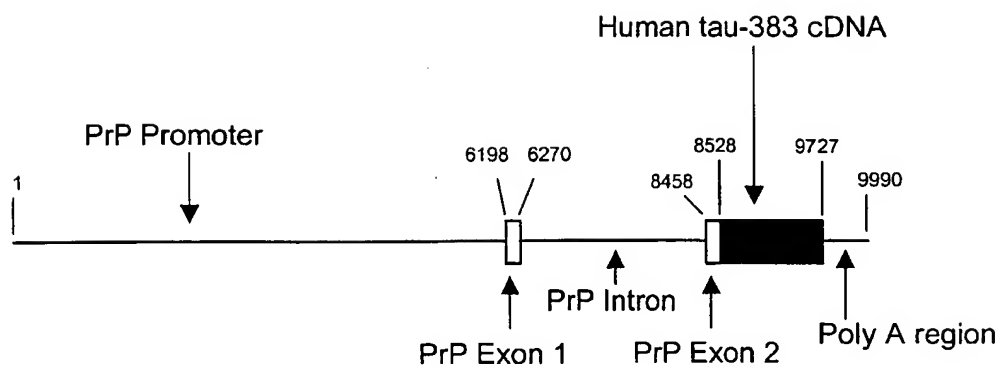
a) administering the drug to a transgenic mouse, the transgenic mouse comprising a transgene, said transgene comprising a polynucleotide encoding a human tau protein operably linked to at least a portion of a regulatory region of a mouse prion gene, wherein the transgenic mouse expresses hyperphosphorylated human tau protein; and

b) comparing the state of phosphorylation of tau in a second transgenic mouse to which the drug was not administered to the state of phosphorylation of tau in the transgenic mouse to which the drug has been administered, wherein a difference in phosphorylation indicates the drug modulates hyperphosphorylation of tau.

15. A method of screening for a drug for treatment of a neurodegenerative disease comprising the step of administering the
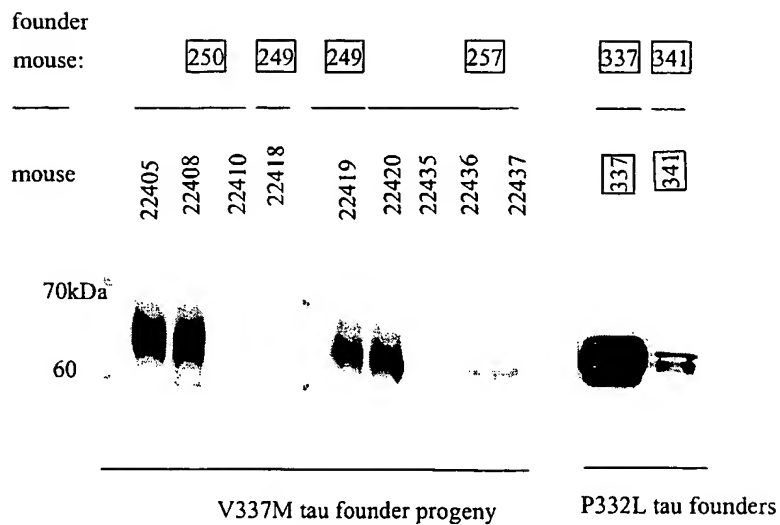
drug to a transgenic mouse which exhibits neurodegenerative disease characteristics, the transgenic mouse comprising a transgene, said transgene comprising a polynucleotide encoding a human tau protein operably linked to at least a portion of a

5   regulatory region of a mouse prion gene, wherein the transgenic mouse expresses human tau protein, and wherein it is determined whether the drug at least partially abates at least one of the characteristics of the disease.

10   16.   A method of screening for a drug that blocks hyperphosphorylation of tau comprising the step of administering the drug to a transgenic mouse, the transgenic mouse comprising a transgene, said transgene comprising a polynucleotide encoding a human tau protein operably linked to at least a portion of a regulatory region of a mouse prion

15   gene, wherein the transgenic mouse expresses hyperphosphorylated human tau protein, and wherein it is determined whether the drug at least partially blocks hyperphosphorylation of tau in the transgenic mouse.

20   17.   A method of screening for a drug that blocks formation of filamentous aggregates of tau comprising the step of administering the drug to a transgenic mouse, the transgenic mouse comprising a transgene, said transgene comprising a polynucleotide encoding a human tau protein operably linked to at least a portion of a

25   regulatory region of a mouse prion gene, wherein the transgenic mouse expresses human tau protein forming filamentous aggregates, and wherein it is determined whether the drug at least partially blocks formation of filamentous aggregates of tau in the transgenic mouse.

30

22

Figure 1

PrP Promoter

Human tau-383 cDNA

6198 6270

8528

8458   9727

1   9990

PrP Exon 1   PrP Intron   PrP Exon 2   Poly A region

Figure 2

wildtype tau founder progeny

V337M tau founder progeny   P332L tau founders

99trans2.ppt
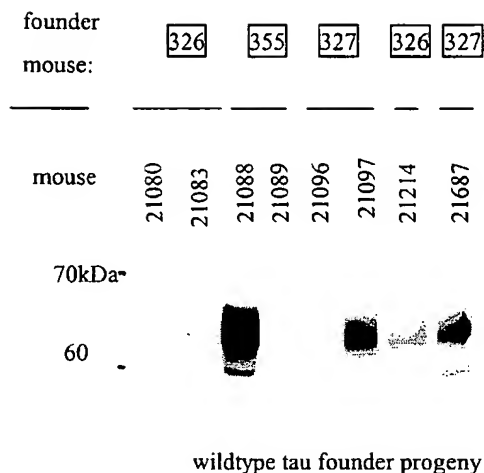
1
SEQUENCE LISTING

<110> Pharmacia & Upjohn Company

<120> Transgenic Mouse Model Of Human Neurodegenerative Disease

<130> PHRM0301

<150> 60/177,319

<151> 2000-01-21

<160> 15

<170> PatentIn version 3.0

<210> 1

<211> 1152

<212> DNA

<213> Homo sapiens

<400> 1
atggctgagc cccgccagga gttcgaagtg atggaagatc acgctgggac gtacgggttg   60

ggggacagga aagatcaggg gggctacacc atgcaccaag accaagaggg tgacacggac   120

gctggcctga agctgaaga agcaggcatt ggagacaccc ccagcctgga agacgaagct   180

gctggtcacg tgacccaagc tcgcatggtc agtaaaagca agacgggac tggaagcgat   240

gacaaaaaag ccaaggggc tgatggtaaa acgaagatcg ccacaccgcg gggagcagcc   300

cctccaggcc agaagggcca ggccaacgcc accaggattc agcaaaaac cccgcccgct   360

ccaaagacac acccagctc tggtgaacct ccaaaatcag gggatcgcag cggctacagc   420

agccccggct ccccaggcac tcccggcagc cgctcccgca ccccgtccct tccaacccca   480

cccacccggg agcccaagaa ggtggcagtg gtccgtactc acccaagtc gccgtcttcc   540

gccaagagcc gcctgcagac agccccgtg cccatgccag acctgaagaa tgtcaagtcc   600

2

```
aagatcggct ccactgagaa cctgaagcac cagccgggag gcgggaaggt gcagataatt    660
aataagaagc tggatcttag caacgtccag tccaagtgtg gctcaaagga taatatcaaa    720
cacgtcccgg gaggcggcag tgtgcaaata gtctacaaac cagttgacct gagcaaggtg    780
acctccaagt gtggctcatt aggcaacatc catcataaac aggaggtgg ccaggtggaa     840
gtaaaatctg agaagcttga cttcaaggac agagtccagt cgaagattgg gtccctggac    900
aatatcaccc acgtccctgg cggaggaaat aaaaagattg aaacccacaa gctgaccttc    960
cgcgagaacg ccaaagccaa gacagaccac ggggcggaga tcgtgtacaa gtcgccagtg    1020
gtgtctgggg acacgtctcc acggcatctc agcaatgtct cctccaccgg cagcatcgac    1080
atggtagact cgccccagct cgccacgcta gctgacgagg tgtctgcctc cctggccaag    1140
cagggtttgt ga                                                        1152
```

<210> 2

<211> 9990

<212> DNA

<213> Mus musculus

<400> 2
```
ggcggccgcg acggatccaa aggcagcaaa aaggcagaga gggtgatact gggcctggct     60
taagcatttg aaacttcaaa gctcacccc aattacacac ttcttccaac aagtccacac      120
ctcctaatta gtgccactct ctgtgggcct acggagagta ttttcattct aactaccaca     180
gttgctgagg aatttaatta aaactacaac cttatcccaa cctagatctt tcagcctttc     240
tgtactacca gagaggggtc atacagcatt gttgtgactc ccattataac ttaaagggaa     300
gctcacacaa agtccagagc cctccatacc ctgcaaatga agaagtacgt tctcaaatcc     360
cttggagcag ggccccactt tggcggcaca aactttaatt tctagacgga acggcatctc     420
tacagaaaga aaagccatgg tatctgcatg ataagtctga aaaggacctg ggcaaatctg     480
cagctgacaa ttccagccat tgctgccact gcgagaaaac cctgctgatg gcagcattgt     540
cagcatcatc tcctccagga acaccggcca tcgagccacg aggacaattg ctgctgctgg     600
agtcaattca tctgccagcc acatcatact ctgggaccgt cactaaccag atccaagcag     660
ccttgaggaa gcatgtcttc tggtggtgac tgatcccaag ggctgacaac aaggtcctca     720
cagaggcatc ttatgtcaac ctatctacca tgcacggtat aagacacatt ctcctctgtg     780
ctgtgtggac actgccatca cacgcaacag aaaggaaact cactcactgt gtctgatgtg     840
```

3

```
gtggtgcttg ttaggggagt tctgggcatg tatggcacca tcgcccatga ggactcctgt   900

ggggtcatgc ccactctact cctctagaga ccatgaagag atggagaggg aagagcaagc   960

acagatgaca ggctagaact aaagaggagt gtcaggtgag cggacctgaa ctcacggctg   1020

ctcagcctga agtggtgtgg ccatctgcat ctggtatctg gtctgaaggt gcgtggatac   1080

cctctgtgcc cgtccagaag tttcctactg aagacagaaa tgcctgtcca gtcatggaag   1140

aatgattggc agttcccact tctcagacca ctgaatgggt cagaacaact actgggtgac   1200

cctaaggtat tcttcagcag atatgtgtga aaaatggaaa gaagatgggt agaaataaac   1260

ggttttagag gaaaaaaact ctcacaaaga tattataaaa agaaagagc tttattattg   1320

agcaagcatt caaccagaat gcacaccaca ggcagtctgc taagggagtg tgcagacagg   1380

aggagtgtcg ccctttatgt gagccagtag ataaggatgc tgtgcgtgtt tttagtaact   1440

ggtcttcagc ttgacagcac catttatcac atggtttaac ctaaattcat ctggcgaatg   1500

aggctgtcac gtacttcctg attagcttta tctgaaatga gacaagcttc acatgttcac   1560

ggcaggaggt aatcctgctg cttagagaac agggtccatc caagccaggc tccttctccc   1620

accaacacgg gtggttgaag agctatctct ccctggtgtg tgtgtttcag agatggctcc   1680

caggtttttg gtttggtttg aattgggttt tggtttttctt actctagccc agactagctt   1740

ggaattctct ggaaagctgc aacggggagc tcaggttcag tgagagatcc tgtctcaaaa   1800

agcagggtga gaagtgattg aggaagacac cccagtgtta acctctgacc tccatatgtg   1860

catgcatgga cacgcatgga tacacataca cacacacaca cacacacaca cacacacaca   1920

cacacacaca cacacacaaa accagaaaga atgaacgccc ccctcccagc ttgtttacag   1980

tagatacaga gcactcgtaa aacatggggt gtaaactgaa tgctgagagt aacttagatg   2040

agtaattaag gaaggaagag gaaagaaacc aggaaaccga gagcaagtga ctggaagatc   2100

gttaggcaat ctccacaccc tgctcgttga agttggaatg ctttcttctt ctgcctcttg   2160

aagttcttta gaagtgctag gatttcacaa ttagtctgtg gtggtttcaa tatgcttcac   2220

ccgtggtaag tggcactatt aggaaacgtg tccttgttga aggaagggtg tcactgcata   2280

ggcgggcttt gaggtgtctt ccagtgctca agctcctccc agtgcaagag aggcagacac   2340

ctgttgcctg cagaagacag tctcctgctg cctttgaatc aagatgtaga actcaagccc   2400

catgtctgcc tgaacctctg aaactgtaag ccagccccaa ttaaatgttt tctttcacaa   2460

gagttgcctt ggtcatggtg tctgttcaca gcaataaaac cctaactaag acagtcttaa   2520

atcaatgaaa agacctttaa ttattcattg aacaaacacc attttcttgt atcaagttgg   2580

cagtgactag taagcaacta tagttctgca ccagggacct ttttggagaa atataccgat   2640
```

4

```
ccaagcatgt tggcatctag attccaaagc caagacacct gccacaccct tccatgcctt   2700

gggttcctgg cagggcatct ggcttcgggg atgtgtattc caggcaccca ctggaatgca   2760

tggaaacaat taaaatagca tcatagaaga cattgcaatc ctagggagaa actataccaa   2820

aactcagaac tatacctggt taagtgtaga aaagacgaaa ggaataaaac caggaatatt   2880

ttaaaatatt tttattgagc tcatgtgcat gggtattttg cctgaaagta tgtctgtgta   2940

ccacatgcat ggctggctcc tgcagaggcc aaaagagagc atcagatctc ctagagctgg   3000

agtttcagaa gtttgtgagc taccacatgg gtgctggaaa caaaacccag gacatctgga   3060

agagcagcca gtgttcttaa ctactgagcc atcattcagg tcccaccatg aatgtttttc   3120

tttattcttc tctatatttt ctaatgtttt tattggaaat atacaacttt gccacacat    3180

aacaaatgac caaagaaatg aggtgagagg ggcagctgtt caaatgctgc ctgggaaggc   3240

ttggccagcc ctggcttggc tgcccctggc tcagctggcc ctgacttggc tgtcccggtg   3300

ccagctgtca tctactgctt cataataagc tgcactttgg gctgaagggg tggctcagcc   3360

tttaaaggct aggctcataa ccaaagtaag ttgcatttta tttgcactag gttgaagggg   3420

gatctgaaac ttgctgtcaa tgttataaaa cattttatct tcaaatttgg tatagggggtc   3480

atagaccaaa ggttctataa accccagaac agcaccactc cctagaaata agcacccata   3540

caagagccta tgggacactt tatagccaaa caaaaagcta tgtttgaaac ttcctttaca   3600

agggcctgag tcccattcat aagggaagga gccccacttc gtaataacac cccactggtg   3660

acatttgaag gggacacatt caaactgtaa caccatctta tatcatttgc acattagggt   3720

caaactgtgc cacgttgtca tttctaagaa gacagaagtt gtcaagcctg tgctttgagc   3780

cacaagtgtg acaacctact ttcaggcaag tcgctacttc cctaagactc taccccaata   3840

ggcctggggt ctggaatgtg tttaacacag atgcaggctt ctgccttagt gcaggcttga   3900

gttctcatgt ccctctctct ttagctttcc gtctcaaggc gcctctcctt agcagaaaaa   3960

atcagaggca taaagcatac atcaggggga agccagagtt ttcagaggga gttttgtgat   4020

ggccttttca gagcattctt gtcaagacta gtttgcctcg ttctctttat taaatgaaag   4080

aaaaataatg cagtgttgca aattagcttt ggtaatggct ccaaccattg tcaggttcac   4140

agtctcattc cgccattcaa aacaacaaac ccaccacact ctctatgcag tgccgtaact   4200

cagaacagcc accaaacagc agaaagaggc tccccgactc ctctcagcct tgccataaac   4260

tcgccggcca catgcttatt ttaaattatt taaattatgt cgtttctccc aacaatgacc   4320

tcccaagtgc ttggttgaca ggcttatacc attaagccga ggcttgcata gcaacgataa   4380

ccaggtaggc tattattata accaggtagc tgccgagcta ctggtcggtc cccttttgtc   4440
```

5

```
tctagaaacc tctcaacccc cacccaaaaa agcttttatt gccacttcct agtgggtaga   4500

gagcagtcag ccaatagata tttgattctt tgaggaaaaa gctgagtttt gatgtctttt   4560

aatcaagcct ttcagagtcc ctctgtgggg gaggccaggt ggaagcgggg tgggaagctg   4620

gtcccttacc taagctaatc tagacaccct cccactcctc ccctgccctc ttgacagatg   4680

cagtcatcct gatcacaatg agtattctct gaggcaggaa ggcaaggctc tggaagatgg   4740

tcaatgcctt cattaagaac ccagagtaaa ggtcaagcag acaccagcac cgctgaaatc   4800

taatttcact gtaattgaat catctcagcc aaaggctgta ttttccagcc ctctcgtggc   4860

ctcttcccca acaactgtca acaactgtgt gagcctaccc atgtatgcgc gctacacac   4920

acacacacac acacacacac acacacacac agggtggggg gacacaatga ttacacaaga   4980

gtacttaata aacaactata attctcctgg ctcggatagt tccttaccac cctctcctcc   5040

tggatccgga tcctaatact ggatacaaat atttaatcca aacccaatct tgtgtctgtt   5100

aatgatcttc agtgtctcgc cctcagcaag aggacaggat attatgtttt ccctgtgatt   5160

tatgacctct tctgtctcag tatcggcagc aatttatta catggctttg gagtgtgtta   5220

tatgtgtagt atggacatga gggtgcatgt caacctatgt gtggaggcca gaggtcaatg   5280

tcatgtcttc cccaatcact gcccagtggt ccctggattc caaactcagg tcctcacgct   5340

tgggaactga gccagtgccc cagctcctaa ccctcccctg ttttaaaaag gtctcattat   5400

gttgcccagg tcagccttga acttgagagt ctcctgactg caggctttca cctgtccaag   5460

tcagcaggca tcttgaacaa gaacatcatt tcctttaagc tgtttcaggc tgtgtttggt   5520

gggagctgtt aaatgcagtg cattttttcc tttggacaca ataaaagaaa aaagtgatta   5580

aatgagttgg gtgtggtggt gcgagtctac aatcctagaa ctcaggagat tgagggagaa   5640

gcattgctct gagtttgagg tcagcttaaa ttacttagta ggaacaccag gccaaattgg   5700

gctatgggat tgtctccaaa gataaagaaa aaagggaagg agagaaaaga aaaagaaagg   5760

aaagaagggg aaaagaagga atcagcagag aataaataag tcaacatgca atggccaata   5820

tactttctag gcctctaatt cttttatagt ttgtgggaaa atgtcgaaaa tcttcgttac   5880

caatttcttg ttaccaaagt tcaacgatgg cttcctcgct ccgttaggta acctttcatt   5940

ttctcaacta cccattatgt aacgggagca ttgggtactg gatcagtctt ccattaaaga   6000

tgattttat agttgctgag cgtcgtcagg gagtgctgac actggggggcg gtttaaacag   6060

atacaagcat ttaagccagt ccggagcggt gactcatccc ccccaccccc cacccccccg   6120

cgagagacgc ggcgcggcca ttggtgagca tcacgccccg ccctcgccc cgcctagttc   6180

ccgcctgccc cgcccctttc cactcccggc tccccgcgt tgtcggatca gcagaccgat   6240
```

6

```
tctgggcgct gcgtcgcatc ggtggcaggt aagcgggctg ctgaagccag gccttggcga      6300

gcactcagcc ttccgtcgtc aagctcggct cactgcgcct ctcggggcct tgaggccacg      6360

gggactagga ctgggactgg gactggggct gagtctggct gggaggtgac tgtacacccc      6420

ctgctgcgcg actcctggag gaaccgaatc ccagggcagc caggccggga gccagccttt      6480

ccttcccgag ccagattcac agctcagcat cgctggggat ggggtggca tcttttgact      6540

gtccttggct gttttcttct ctctttgtag tagctacagc gaacataatt ttacctcgtt      6600

attccaccac agtcattact cccttgcaca gtttcattct caacgtcgcc gtgcgccttc      6660

actgccctgt ctaggcgttt tcatgattgt ctattttctt gtactttgaa taccgtggtt      6720

taatagcagt tgcgggtgcg cagaattctc catttcctta agagaaactc ctgggagaat      6780

gggactaaag acgtgcaaat ttaattatat cgcaaacagg aatcaaaatt ttgcattaaa      6840

atgccaaaca tcttgaaaaa ttaactattc aatgaagaaa aggaactact ttacctacac      6900

acacatccga gagcttcgag gaggcgaagg aaatagaaag ctaagggatg atttgggttg      6960

tatttgaatc tgacacaagc tttccatatt atttatagca gggactaaac gatgagtcat      7020

tttctgaata agatgcaaat taaagcaagt ttgtttgttg tctttacatc tattaaatag      7080

acagagacaa tggcaacagc aaccctaacc tagaggttgc ctgaaagtgt caggtttggg      7140

aacaagtggc cctgcttaag ggctagaaag attgctttac aaccaacaat catgacttga      7200

cattgcctgg ggttcctttt gtctattcct tttttaaaag actagtgttt attttatgtg      7260

catgagtgtt ttgcatccac attcgcctgt atacacacct ggttctgtgg aggtcaggag      7320

agggtgctgg atgccctggc actagagcct tggatggtta tgtgagcccc tgccacaggg      7380

gagctcagaa ccaaatccag gtcctctgga agagcaacca gagctcttaa aacttctaag      7440

tatccctcca tcccctttcc atcatatttg gaaaggagaa aactgctacc catgcctggc      7500

atttatttca gagattaact gtctgtgtaa aacttgacat tgaaagtgca ctattctgtt      7560

tcccattcat acttagttga gactactgta agtcagttag gcttttttt gtttggttcc      7620

ttggttagtt tggagtgtgt ttgtgagctc attaacaggc tttcaatatg tagctggaat      7680

ttgctgtgta gaccagacag gcctcaaatt tgtggcaatc ctccctgcat cttcccagaa      7740

tgccctggta caggcataaa ccaccgtgcc cagcagtaaa acaatctggt gaggtattat      7800

tagtcgtgtg ctgtgaccca gaaaccccac tcctggcaat ttactgggaa ggaacaaaca      7860

aagggctagg ggagccatat ggcctgcagt tagagaaaat tagatccaac tgaaaaatca      7920

acctaaaggt gtaaaagcca agcagttaag aaactgacaa gctcatgatg gaagccgagg      7980

ccatcgtgaa cactcttcat tttaggcccc acgtatcact ggggacaact gagagtcaaa      8040
```

```
gtacaggtaa ggagaccaag gcttttcagg actcaggctg tctcagtgaa aagcccagaa    8100

gagcagtaat tgaaagagct cagacgatgt gtctgatctc ccctgtttgt ttgttgctgt    8160

attatttcca ctaacttatt tgggaggaaa aaaaacagtt cacaggcttc ttttcttgaa    8220

atactgggga ttgctgggat cgaacccagg gataggtttt tagtttctaa aataacatag    8280

atcatgccct gtttgctttt tggaatatgt ttgcgctgcc cttattttca tgttcaaata    8340

ctgctccatt ttgcgtgact ctttagtatt ggtttgatga tttgcatatt agattagatt    8400

gtatttcagt tctcagactt atttatcaat tctagttttc tctttttgtt gttttaaagg    8460

actcctgagt atatttcaga actgaaccat ttcaaccgag ctgaagcatt ctgccttcct    8520

agtggtacct cgactatcag gtgaactttg aaccaggatg gctgagcccc gccaggagtt    8580

cgaagtgatg gaagatcacg ctgggacgta cgggttgggg gacaggaaag atcaggggg    8640

ctacaccatg caccaagacc aagagggtga cacggacgct ggcctgaaag ctgaagaagc    8700

aggcattgga gacaccccca gcctggaaga cgaagctgct ggtcacgtga cccaagctcg    8760

catggtcagt aaaagcaaag acgggactgg aagcgatgac aaaaaagcca aggggggctga    8820

tggtaaaacg aagatcgcca caccgcgggg agcagcccct ccaggccaga agggccaggc    8880

caacgccacc aggattccag caaaaacccc gcccgctcca aagacaccac ccagctctgg    8940

tgaacctcca aaatcagggg atcgcagcgg ctacagcagc cccggctccc caggcactcc    9000

cggcagccgc tcccgcaccc cgtcccttcc aaccccaccc acccgggagc caagaaggt    9060

ggcagtggtc cgtactccac ccaagtcgcc gtcttccgcc aagagccgcc tgcagacagc    9120

ccccgtgccc atgccagacc tgaagaatgt caagtccaag atcggctcca ctgagaacct    9180

gaagcaccag ccgggaggcg ggaaggtgca gataattaat aagaagctgg atcttagcaa    9240

cgtccagtcc aagtgtggct caaaggataa tatcaaacac gtcccgggag cggcagtgt    9300

gcaaatagtc tacaaaccag ttgacctgag caaggtgacc tccaagtgtg gctcattagg    9360

caacatccat cataaaccag gaggtggcca ggtggaagta aaatctgaga agcttgactt    9420

caaggacaga gtccagtcga agattgggtc cctggacaat atcacccacg tccctggcgg    9480

aggaaataaa aagattgaaa cccacaagct gaccttccgc gagaacgcca aagccaagac    9540

agaccacggg gcggagatcg tgtacaagtc gccagtggtg tctggggaca cgtctccacg    9600

gcatctcagc aatgtctcct ccaccggcag catcgacatg gtagactcgc cccagctcgc    9660

cacgctagct gacgaggtgt ctgcctccct ggccaagcag ggtttgtgat caggcccctg    9720

gggcggtcaa taattgtgga gaggagagaa tgagagagtg tggaaaaaaa aagaataatg    9780

acccggcccc cgccctctgc ccccagctgc tcctcgcagt tcgggaattc ggatccagat    9840
```

8

```
cttattaaag cagaacttgt ttattgcagc ttataatggt tacaaataaa gcaatagcat   9900

cacaaatttc acaaataaag catttttttc actgcattct agttgtggtt tgtccaaact   9960

catcaatgta tcttatcatg tctggtcgac                                   9990
```

<210> 3

<211> 25

<212> DNA

<213> Artificial

<220>

<223> Primer

```
<400> 3
agtaattgaa agagctcaga cgatg                                           25
```

<210> 4

<211> 23

<212> DNA

<213> Artificial

<220>

<223> Primer

```
<400> 4
tgtcaccctc ttggtcttgg tgc                                             23
```

<210> 5

<211> 22

<212> DNA

<213> Artificial

<220>

<223> Primer

```
<400> 5
gtactccacc caagtcgccg tc                                              22
```

9

<210> 6

<211> 23

<212> DNA

<213> Artificial

<220>

<223> Primer

<400> 6
gcagcagcat cgaagcttct cag   23

<210> 7

<211> 48

<212> DNA

<213> Artificial

<220>

<223> Missense

<400> 7
gcagcagcat cgaagcttct cagattttac ttccatctgg ccacctcc   48

<210> 8

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Primer

<400> 8
ccgccaagag ccgcctgcag   20

<210> 9

<211> 61

<212> DNA

```
<213>  Artificial


<220>

<223>  Primer

<400>  9
gcagcagcat cgaagcttct cagattttac ttccacctgg ccacctccta gtttatgatg    60

g                                                                     61


<210>  10

<211>  1152

<212>  DNA

<213>  Homo sapiens


<400>  10
atggctgagc cccgccagga gttcgaagtg atggaagatc acgctgggac gtacgggttg    60

ggggacagga aagatcaggg gggctacacc atgcaccaag accaagaggg tgacacggac   120

gctggcctga aagctgaaga agcaggcatt ggagacaccc ccagcctgga agacgaagct   180

gctggtcacg tgacccaagc tcgcatggtc agtaaaagca agacgggac tggaagcgat    240

gacaaaaaag ccaaggggc tgatggtaaa acgaagatcg ccacaccgcg gggagcagcc    300

cctccaggcc agaagggcca ggccaacgcc accaggattc agcaaaaac cccgcccgct    360

ccaaagacac cacccagctc tggtgaacct ccaaaatcag gggatcgcag cggctacagc    420

agccccggct ccccaggcac tcccggcagc cgctcccgca ccccgtccct tccaacccca    480

cccacccggg agcccaagaa ggtggcagtg gtccgtactc cacccaagtc gccgtcttcc    540

gccaagagcc gcctgcagac agcccccgtg cccatgccag acctgaagaa tgtcaagtcc    600

aagatcggct ccactgagaa cctgaagcac cagccgggag cgggaaggt gcagataatt    660

aataagaagc tggatcttag caacgtccag tccaagtgtg gctcaaagga taatatcaaa    720

cacgtcccgg gaggcggcag tgtgcaaata gtctacaaac cagttgacct gagcaaggtg    780

acctccaagt gtggctcatt aggcaacatc catcataaac caggaggtgg ccagatggaa    840

gtaaaatctg agaagcttga cttcaaggac agagtccagt cgaagattgg tcccctggac    900

aatatcaccc acgtccctgg cggaggaaat aaaaagattg aaacccacaa gctgaccttc    960

cgcgagaacg ccaaagccaa gacagaccac ggggcggaga tcgtgtacaa gtcgccagtg   1020

gtgtctgggg acacgtctcc acggcatctc agcaatgtct cctccaccgg cagcatcgac   1080
```

11

atggtagact cgccccagct cgccacgcta gctgacgagg tgtctgcctc cctggccaag   1140

cagggtttgt ga   1152

<210> 11

<211> 1152

<212> DNA

<213> Homo sapiens

<400> 11

atggctgagc cccgccagga gttcgaagtg atggaagatc acgctgggac gtacgggttg   60

ggggacagga aagatcaggg gggctacacc atgcaccaag accaagaggg tgacacggac   120

gctggcctga aagctgaaga agcaggcatt ggagacaccc ccagcctgga agacgaagct   180

gctggtcacg tgacccaagc tcgcatggtc agtaaaagca agacgggac tggaagcgat   240

gacaaaaaag ccaagggggc tgatggtaaa acgaagatcg ccacaccgcg gggagcagcc   300

cctccaggcc agaagggcca ggccaacgcc accaggattc agcaaaaac cccgcccgct   360

ccaaagacac cacccagctc tggtgaacct ccaaaatcag gggatcgcag cggctacagc   420

agccccggct ccccaggcac tcccggcagc cgctcccgca ccccgtccct tccaacccca   480

cccacccggg agcccaagaa ggtggcagtg gtccgtactc cacccaagtc gccgtcttcc   540

gccaagagcc gcctgcagac agcccccgtg cccatgccag acctgaagaa tgtcaagtcc   600

aagatcggct ccactgagaa cctgaagcac cagccgggag cgggaaggt gcagataatt   660

aataagaagc tggatcttag caacgtccag tccaagtgtg gctcaaagga taatatcaaa   720

cacgtcccgg gaggcggcag tgtgcaaata gtctacaaac cagttgacct gagcaaggtg   780

acctccaagt gtggctcatt aggcaacatc catcataaac taggaggtgg ccaggtggaa   840

gtaaaatctg agaagcttga cttcaaggac agagtccagt cgaagattgg gtccctggac   900

aatatcaccc acgtccctgg cggaggaaat aaaaagattg aaacccacaa gctgaccttc   960

cgcgagaacg ccaaagccaa gacagaccac ggggcggaga tcgtgtacaa gtcgccagtg   1020

gtgtctgggg acacgtctcc acggcatctc agcaatgtct cctccaccgg cagcatcgac   1080

atggtagact cgccccagct cgccacgcta gctgacgagg tgtctgcctc cctggccaag   1140

cagggtttgt ga   1152

<210> 12

<211> 19

```
<212>   DNA

<213>   Artificial


<220>

<223>   Primer

<400>   12
gcattggaga caccccccag                                              19


<210>   13

<211>   21

<212>   DNA

<213>   Artificial


<220>

<223>   Primer

<400>   13
gcttttactg accatgcgag c                                            21


<210>   14

<211>   25

<212>   DNA

<213>   Artificial


<220>

<223>   Primer

<400>   14
ctggaagacg aagctgctgg tcacg                                        25


<210>   15

<211>   9990

<212>   DNA

<213>   Artificial
```

<220>

<223> PrP/tau transgene construct

<400> 15

```
ggcggccgcg acggatccaa aggcagcaaa aaggcagaga gggtgatact gggcctggct    60

taagcatttg aaacttcaaa gctcaccccc aattacacac ttcttccaac aagtccacac   120

ctcctaatta gtgccactct ctgtgggcct acggagagta ttttcattct aactaccaca   180

gttgctgagg aatttaatta aaactacaac cttatcccaa cctagatctt tcagcctttc   240

tgtactacca gagaggggtc atacagcatt gttgtgactc ccattataac ttaaagggaa   300

gctcacacaa agtccagagc cctccatacc ctgcaaatga agaagtacgt tctcaaatcc   360

cttggagcag ggccccactt tggcggcaca aactttaatt tctagacgga acggcatctc   420

tacagaaaga aaagccatgg tatctgcatg ataagtctga aaaggacctg ggcaaatctg   480

cagctgacaa ttccagccat tgctgccact gcgagaaaac cctgctgatg gcagcattgt   540

cagcatcatc tcctccagga acaccggcca tcgagccacg aggacaattg ctgctgctgg   600

agtcaattca tctgccagcc acatcatact ctgggaccgt cactaaccag atccaagcag   660

ccttgaggaa gcatgtcttc tggtggtgac tgatcccaag ggctgacaac aaggtcctca   720

cagaggcatc ttatgtcaac ctatctacca tgcacggtat aagacacatt ctcctctgtg   780

ctgtgtggac actgccatca cacgcaacag aaaggaaact cactcactgt gtctgatgtg   840

gtggtgcttg ttaggggagt tctgggcatg tatggcacca tcgcccatga ggactcctgt   900

ggggtcatgc ccactctact cctctagaga ccatgaagag atggagaggg aagagcaagc   960

acagatgaca ggctagaact aaagaggagt gtcaggtgag cggacctgaa ctcacggctg  1020

ctcagcctga agtggtgtgg ccatctgcat ctggtatctg gtctgaaggt gcgtggatac  1080

cctctgtgcc cgtccagaag tttcctactg aagacagaaa tgcctgtcca gtcatggaag  1140

aatgattggc agttcccact ctcagacca ctgaatgggt cagaacaact actgggtgac  1200

cctaaggtat tcttcagcag atatgtgtga aaaatggaaa gaagatgggt agaaataaac  1260

ggttttagag gaaaaaaact ctcacaaaga tattataaaa agaaagagc tttattattg  1320

agcaagcatt caaccagaat gcacaccaca ggcagtctgc taagggagtg tgcagacagg  1380

aggagtgtcg ccctttatgt gagccagtag ataaggatgc tgtgcgtgtt tttagtaact  1440

ggtcttcagc ttgacagcac catttatcac atggtttaac ctaaattcat ctggcgaatg  1500

aggctgtcac gtacttcctg attagcttta tctgaaatga acaagcttc acatgttcac  1560

ggcaggaggt aatcctgctg cttagagaac agggtccatc caagccaggc tccttctccc  1620

accaacacgg gtggttgaag agctatctct ccctggtgtg tgtgtttcag agatggctcc  1680
```

14

```
caggtttttg gtttggtttg aattgggttt tggttttctt actctagccc agactagctt   1740

ggaattctct ggaaagctgc aacggggagc tcaggttcag tgagagatcc tgtctcaaaa   1800

agcagggtga gaagtgattg aggaagacac cccagtgtta acctctgacc tccatatgtg   1860

catgcatgga cacgcatgga tacacataca cacacacaca cacacacaca cacacacaca   1920

cacacacaca cacacacaaa accagaaaga atgaacgccc ccctcccagc ttgtttacag   1980

tagatacaga gcactcgtaa aacatggggt gtaaactgaa tgctgagagt aacttagatg   2040

agtaattaag gaaggaagag gaaagaaacc aggaaaccga gagcaagtga ctggaagatc   2100

gttaggcaat ctccacaccc tgctcgttga agttggaatg ctttcttctt ctgcctcttg   2160

aagttcttta gaagtgctag gatttcacaa ttagtctgtg gtggtttcaa tatgcttcac   2220

ccgtggtaag tggcactatt aggaaacgtg tccttgttga aggaagggtg tcactgcata   2280

ggcgggcttt gaggtgtctt ccagtgctca agctcctccc agtgcaagag aggcagacac   2340

ctgttgcctg cagaagacag tctcctgctg cctttgaatc aagatgtaga actcaagccc   2400

catgtctgcc tgaacctctg aaactgtaag ccagccccaa ttaaatgttt tctttcacaa   2460

gagttgcctt ggtcatggtg tctgttcaca gcaataaaac cctaactaag acagtcttaa   2520

atcaatgaaa agacctttaa ttattcattg aacaaacacc attttcttgt atcaagttgg   2580

cagtgactag taagcaacta tagttctgca ccagggacct ttttggagaa atataccgat   2640

ccaagcatgt tggcatctag attccaaagc caagacacct gccacaccct tccatgcctt   2700

gggttcctgg cagggcatct ggcttcgggg atgtgtattc caggcaccca ctggaatgca   2760

tggaaacaat taaaatagca tcatagaaga cattgcaatc ctagggagaa actataccaa   2820

aactcagaac tatacctggt taagtgtaga aaagacgaaa ggaataaaac caggaatatt   2880

ttaaaatatt tttattgagc tcatgtgcat gggtattttg cctgaaagta tgtctgtgta   2940

ccacatgcat ggctggctcc tgcagaggcc aaaagagagc atcagatctc ctagagctgg   3000

agtttcagaa gtttgtgagc taccacatgg gtgctggaaa caaaacccag gacatctgga   3060

agagcagcca gtgttcttaa ctactgagcc atcactcagg tcccaccatg aatgtttttc   3120

tttattcttc tctatatttt ctaatgtttt tattggaaat atacaacttt tgccacacat   3180

aacaaatgac caaagaaatg aggtgagagg ggcagctgtt caaatgctgc ctgggaaggc   3240

ttggccagcc ctggcttggc tgccctggc tcagctggcc ctgacttggc tgtcccggtg    3300

ccagctgtca tctactgctt cataataagc tgcactttgg gctgaagggg tggctcagcc   3360

tttaaaggct aggctcataa ccaaagtaag ttgcatttta tttgcactag gttgaagggg   3420

gatctgaaac ttgctgtcaa tgttataaaa cattttatct tcaaatttgg tataggggtc   3480
```

15

```
atagaccaaa ggttctataa accccagaac agcaccactc cctagaaata agcacccata   3540

caagagccta tgggacactt tatagccaaa caaaaagcta tgtttgaaac ttcctttaca   3600

agggcctgag tcccattcat aagggaagga gccccacttc gtaataacac cccactggtg   3660

acatttgaag gggacacatt caaactgtaa caccatctta tatcatttgc acattagggt   3720

caaactgtgc cacgttgtca tttctaagaa gacagaagtt gtcaagcctg tgctttgagc   3780

cacaagtgtg acaacctact ttcaggcaag tcgctacttc cctaagactc tacccaata   3840

ggcctggggt ctggaatgtg tttaacacag atgcaggctt ctgccttagt gcaggcttga   3900

gttctcatgt ccctctctct ttagctttcc gtctcaaggc gcctctcctt agcagaaaaa   3960

atcagaggca taaagcatac atcaggggga agccagagtt ttcagaggga gttttgtgat   4020

ggccttttca gagcattctt gtcaagacta gtttgcctcg ttctctttat aaatgaaag   4080

aaaaataatg cagtgttgca aattagcttt ggtaatggct ccaaccattg tcaggttcac   4140

agtctcattc cgccattcaa aacaacaaac ccaccacact ctctatgcag tgccgtaact   4200

cagaacagcc accaaacagc agaaagaggc tccccgactc ctctcagcct tgccataaac   4260

tcgccggcca catgcttatt ttaaattatt taaattatgt cgtttctccc aacaatgacc   4320

tcccaagtgc ttggttgaca ggcttatacc attaagccga ggcttgcata gcaacgataa   4380

ccaggtaggc tattattata accaggtagc tgccgagcta ctggtcggtc cccttttgtc   4440

tctagaaacc tctcaacccc cacccaaaaa agcttttatt gccacttcct agtgggtaga   4500

gagcagtcag ccaatagata tttgattctt tgaggaaaaa gctgagtttt gatgtctttt   4560

aatcaagcct ttcagagtcc ctctgtgggg gaggccaggt ggaagcgggg tgggaagctg   4620

gtcccttacc taagctaatc tagacaccct cccactcctc ccctgccctc ttgacagatg   4680

cagtcatcct gatcacaatg agtattctct gaggcaggaa ggcaaggctc tggaagatgg   4740

tcaatgcctt cattaagaac ccagagtaaa ggtcaagcag acaccagcac cgctgaaatc   4800

taatttcact gtaattgaat catctcagcc aaaggctgta ttttccagcc ctctcgtggc   4860

ctcttcccca acaactgtca acaactgtgt gagcctaccc atgtatgcgc gctcacacac   4920

acacacacac acacacacac acacacacac agggtggggg gacacaatga ttacacaaga   4980

gtacttaata aacaactata attctcctgg ctcggatagt tccttaccac cctctcctcc   5040

tggatccgga tcctaatact ggatacaaat atttaatcca aacccaatct tgtgtctgtt   5100

aatgatcttc agtgtctcgc cctcagcaag aggacaggat attatgtttt ccctgtgatt   5160

tatgacctct tctgtctcag tatcggcagc aatttattta catggctttg gagtgtgtta   5220

tatgtgtagt atggacatga gggtgcatgt caacctatgt gtggaggcca gaggtcaatg   5280
```

16

```
tcatgtcttc cccaatcact gcccagtggt ccctggattc caaactcagg tcctcacgct   5340

tgggaactga gccagtgccc cagctcctaa ccctcccctg ttttaaaaag gtctcattat   5400

gttgcccagg tcagccttga acttgagagt ctcctgactg caggctttca cctgtccaag   5460

tcagcaggca tcttgaacaa gaacatcatt tcctttaagc tgtttcaggc tgtgtttggt   5520

gggagctgtt aaatgcagtg cattttttcc tttggacaca ataaagaaa aaagtgatta   5580

aatgagttgg gtgtggtggt gcgagtctac aatcctagaa ctcaggagat tgagggagaa   5640

gcattgctct gagtttgagg tcagcttaaa ttacttagta ggaacaccag gccaaattgg   5700

gctatgggat tgtctccaaa gataaagaaa aaagggaagg agagaaaaga aaaagaaagg   5760

aaagaagggg aaaagaagga atcagcagag aataaataag tcaacatgca atggccaata   5820

tactttctag gcctctaatt cttttatagt ttgtgggaaa atgtcgaaaa tcttcgttac   5880

caatttcttg ttaccaaagt tcaacgatgg cttcctcgct ccgttaggta acctttcatt   5940

ttctcaacta cccattatgt aacgggagca ttgggtactg gatcagtctt ccattaaaga   6000

tgatttttat agttgctgag cgtcgtcagg gagtgctgac actgggggcg gtttaaacag   6060

atacaagcat ttaagccagt ccggagcggt gactcatccc cccccacccc cacccccccg   6120

cgagagacgc ggcgcggcca ttggtgagca tcacgccccg cccctcgccc cgcctagttc   6180

ccgcctgccc cgccccttc cactcccggc tcccccgcgt tgtcggatca gcagaccgat    6240

tctgggcgct gcgtcgcatc ggtggcaggt aagcgggctg ctgaagccag gccttggcga   6300

gcactcagcc ttccgtcgtc aagctcggct cactgcgcct ctcggggcct tgaggccacg   6360

gggactagga ctgggactgg gactggggct gagtctggct gggaggtgac tgtacacccc   6420

ctgctgcgcg actcctggag gaaccgaatc ccagggcagc caggccggga gccagccttt   6480

ccttcccgag ccagattcac agctcagcat cgctggggat ggggtggca tcttttgact    6540

gtccttggct gttttcttct ctctttgtag tagctacagc gaacataatt ttacctcgtt   6600

attccaccac agtcattact cccttgcaca gtttcattct caacgtcgcc gtgcgccttc   6660

actgccctgt ctaggcgttt tcatgattgt ctattttctt gtactttgaa taccgtggtt   6720

taatagcagt tgcgggtgcg cagaattctc catttcctta agagaaactc ctgggagaat   6780

gggactaaag acgtgcaaat ttaattatat cgcaaacagg aatcaaaatt ttgcattaaa   6840

atgccaaaca tcttgaaaaa ttaactattc aatgaagaaa aggaactact ttacctacac   6900

acacatccga gagcttcgag gaggcgaagg aaatagaaag ctaagggatg atttgggttg   6960

tatttgaatc tgacacaagc tttccatatt atttatagca gggactaaac gatgagtcat   7020

tttctgaata agatgcaaat taaagcaagt ttgtttgttg tctttacatc tattaaatag   7080
```

```
acagagacaa tggcaacagc aaccctaacc tagaggttgc ctgaaagtgt cacgtttggg    7140

aacaagtggc cctgcttaag ggctagaaag attgctttac aaccaacaat catgacttga    7200

cattgcctgg ggttcctttt gtccattcct tttttaaaag actagtgttt atcctatgtg    7260

catgagtgtt ttgcatccac attcgcctgt atacacacct ggttctgtgg aggtcaggag    7320

agggtgctgg atgccctggc actagagcct tggatggtta tgtgagcccc tgccacaggg    7380

gagctcagaa ccaaatccag gtcctctgga agagcaacca gagctcttaa aacttctaag    7440

tatccctcca tccccttttcc atcatatttg gaaaggagaa aactgctacc catgcctggc    7500

atttatttca gagattaact gtctgtgtaa aacttgacat tgaaagtgca ctattctgtt    7560

tcccattcat acttagttga gactactgta agtcagttag ggcttttttt gtttggttcc    7620

ttggttagtt tggagtgtgt ttgtgagctc attaacaggc tttcaatatg tagctggaat    7680

ttgctgtgta gaccagacag gcctcaaatt tgtggcaatc ctccctgcat cttcccagaa    7740

tgccctggta caggcataaa ccaccgtgcc cagcagtaaa acaatctggt gaggtattat    7800

tagtcgtgtg ctgtgaccca gaaaccccac tcctggcaat ttactgggaa ggaacaaaca    7860

aagggctagg ggagccatat ggcctgcagt tagagaaaat tagatccaac tgaaaaatca    7920

acctaaaggt gtaaaagcca agcagttaag aaactgacaa gctcatgatg gaagccgagg    7980

ccatcgtgaa cactcttcat tttaggcccc acgtatcact ggggacaact gagagtcaaa    8040

gtacaggtaa ggagaccaag gcttttcagg actcaggctg tctcagtgaa aagcccagaa    8100

gagcagtaat tgaaagagct cagacgatgt gtctgatctc ctctgtttgt ttgttgctgt    8160

attatttcca ctaacttatt tgggaggaaa aaaaacagtt cacaggcttc ttttcttgaa    8220

atactgggga ttgctgggat cgaacccagg gataggtttt tagtttctaa aataacatag    8280

atcatgccct gtttgctttt tggaatatgt ttgcgctgcc cttattttca tgttcaaata    8340

ctgctccatt ttgcgtgact ctttagtatt ggtttgatga tttgcatatt agattagatt    8400

gtatttcagt tctcagactt atttatcaat tctagttttc tcttttttgtt gttttaaagg    8460

actcctgagt atatttcaga actgaaccat ttcaaccgag ctgaagcatt ctgccttcct    8520

agtggtacct cgactatcag gtgaactttg aaccaggatg gctgagcccc gccaggagtt    8580

cgaagtgatg gaagatcacg ctgggacgta cgggttgggg gacaggaaag atcaggggggg    8640

ctacaccatg caccaagacc aagagggtga cacggacgct ggcctgaaag ctgaagaagc    8700

aggcattgga gacacccccca gcctggaaga cgaagctgct ggtcacgtga cccaagctcg    8760

catggtcagt aaaagcaaag acgggactgg aagcgatgac aaaaaagcca aggggggctga    8820

tggtaaaacg aagatcgcca caccgcgggg agcagcccct ccaggccaga agggccaggc    8880
```

```
caacgccacc aggattccag caaaaacccc gcccgctcca aagacaccac ccagctctgg     8940

tgaacctcca aaatcagggg atcgcagcgg ctacagcagc cccggctccc caggcactcc     9000

cggcagccgc tcccgcaccc cgtcccttcc aaccccaccc acccgggagc ccaagaaggt     9060

ggcagtggtc cgtactccac ccaagtcgcc gtcttccgcc aagagccgcc tgcagacagc     9120

ccccgtgccc atgccagacc tgaagaatgt caagtccaag atcggctcca ctgagaacct     9180

gaagcaccag ccgggaggcg ggaaggtgca gataattaat aagaagctgg atcttagcaa     9240

cgtccagtcc aagtgtggct caaaggataa tatcaaacac gtcccgggag gcggcagtgt     9300

gcaaatagtc tacaaaccag ttgacctgag caaggtgacc tccaagtgtg gctcattagg     9360

caacatccat cataaaccag gaggtggcca ggtggaagta aaatctgaga agcttgactt     9420

caaggacaga gtccagtcga agattgggtc cctggacaat atcacccacg tccctggcgg     9480

aggaaataaa aagattgaaa cccacaagct gaccttccgc gagaacgcca aagccaagac     9540

agaccacggg gcggagatcg tgtacaagtc gccagtggtg tctggggaca cgtctccacg     9600

gcatctcagc aatgtctcct ccaccggcag catcgacatg gtagactcgc cccagctcgc     9660

cacgctagct gacgaggtgt ctgcctccct ggccaagcag ggtttgtgat caggcccctg     9720

gggcggtcaa taattgtgga gaggagagaa tgagagagtg tggaaaaaaa aagaataatg     9780

acccggcccc cgccctctgc ccccagctgc tcctcgcagt tcgggaattc ggatccagat     9840

cttattaaag cagaacttgt ttattgcagc ttataatggt tacaaataaa gcaatagcat     9900

cacaaatttc acaaataaag catttttttc actgcattct agttgtggtt tgtccaaact     9960

catcaatgta tcttatcatg tctggtcgac                                      9990
```